



A sad tale: has the small mussel *Idas argenteus* lost its symbionts?

CLARA F. RODRIGUES^{1,2*}, SVEN R. LAMING^{1,2}, SYLVIE M. GAUDRON¹, GRAHAM OLIVER³, NADINE LE BRIS⁴ and SÉBASTIEN DUPERRON^{1,5}

¹*Sorbonne Universités, UPMC Université Paris 06, UMR7208 BOREA, Laboratoire Biologie des Organismes Aquatiques et Ecosystèmes, 7 quai St Bernard, 75005 Paris, France*

²*Departamento de Biología & CESAM, Universidade de Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal*

³*National Museum of Wales, Cathays Park, CF10 3NP, Cardiff, Wales, UK*

⁴*Sorbonne Universités, UPMC Université Paris 06, CNRS, UMR8222, LECOB (Laboratoire d'Ecogéochimie des Environments Benthiques), F-66650 Banyuls-sur-Mer, France*

⁵*Institut Universitaire de France, Saint-Michel 103, 75005 Paris, France*

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Idas argenteus (Bivalvia: Mytilidae) belongs to a genus of mussels that are often associated with sunken wood and vertebrate bones in the deep sea. By contrast to other species currently included within the genus *Idas* and other related genera, such as *Bathymodiolus*, *I. argenteus* was documented to lack chemosynthetic symbionts bacterial symbionts in its gills. In the present study, new specimens are assigned to *I. argenteus* based on shell and soft parts analysis. Molecular data confirm the absence or low abundance of symbionts. Phylogeny based on five genes indicates that the symbiont-bearing *I. washingtonius* is the closest relative of *I. argenteus*. Symbiosis loss or extreme reduction is thus inferred to have occurred subsequent to the speciation event, 11–13 Mya. This is the first report of a loss of symbiosis within the clade of deep-sea chemosynthetic mussels. © 2014 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2015, **114**, 398–405.

ADDITIONAL KEYWORDS: Bivalvia – ecology – evolution – mytilidae.

INTRODUCTION

Symbiosis between bacteria and metazoans is key to the productivity of deep-sea cold seeps and hydrothermal vents, in which most of the primary production is ensured by chemoautotrophic symbionts (Dubilier, Bergin & Lott, 2008). Symbiosis is usually facultative for the bacteria and several shifts between free-living and symbiotic lifestyles during the life of a bacterium or during evolution of bacterial groups are reported (Dubilier *et al.*, 2008; Duperron *et al.*, 2013; Wentrup *et al.*, 2013). Taxa within the annelids and bivalves, on the other hand, live in obligate symbiosis with bacteria that contribute to their nutrition, and symbiosis is considered as the key synapomorphy that

allowed the efficient colonization of reducing habitats (Won, Jones & Vrijenhoek, 2008; Duperron, 2010). Habitat and depth shifts (and reversals) can occur (Won *et al.*, 2008; Thubaut *et al.*, 2013). It is thus reasonable to propose that symbiosis may disappear or become less significant to the host's metabolism under particular circumstances. Recent examples within the 'bathymodiolin' mussels (a clade within the Mytilidae that includes deep-sea species currently assigned to subfamilies Bathymodiolinae and Modiolinae) indicate the presence of extracellular symbionts, sometimes in low abundances, and possibly intraspecific variability in symbiont types depending on sites (Duperron, 2010; Rodrigues *et al.*, 2013; Thubaut *et al.*, 2013). In at least one case, namely *Idas argenteus* Jeffreys, 1876 from sunken wood in the north Atlantic, microscopy-based evidence supported the absence of bacterial symbionts and a

*Corresponding author. E-mail: clara.rodrigues@ua.pt

larvivorous regime (Ockelmann & Dinesen, 2011). This mytilid is of particular taxonomic importance because it is the type species of its genus (Lorion *et al.*, 2013; Thubaut *et al.*, 2013). Whether *I. argenteus* is a relict species belonging to a deep-branching lineage emerging before the acquisition of symbiosis in bathymodiolin mussels, or whether it belongs to a recently emerging lineage in which symbiosis was secondarily lost, has not been investigated as a result of the lack of material available for molecular studies.

We recently collected specimens resembling *I. argenteus* during colonization experiments deployed in the Lacaze-Duthiers (LD) Canyon (Gulf of Lion, Mediterranean) and at the Rainbow hydrothermal vent site in the Mid-Atlantic Ridge (MAR). Multilocus sequencing was used to determine the phylogenetic position of these specimens. Assignment to *I. argenteus* was based on the comparison of shell and anatomy with the original and subsequent descriptions (Jeffreys, 1876; Dean, 1993; Oliver & Holmes, 2009; Ockelmann & Dinesen, 2011). Polymerase chain reaction (PCR) amplification of bacterial genes was employed to test for the presence of symbionts.

MATERIAL AND METHODS

SAMPLING

Mussels were collected from CHEMECOLI colonization devices deployed for 414 days ($N = 1$) at the Rainbow hydrothermal vent (MAR, 36°13.74'N, 33°54.05'W, 2279 m; Gaudron *et al.*, 2010) and 382 days ($N = 1$) in the LD canyon (42°32.72'N, 03°25.27'E, 513 m). Colonization devices were made of a hollow poly(vinyl chloride) cylinder (diameter 14 cm, height 10 cm, total volume 1.539 dm³). The cylinder was drilled with lateral holes to permit the circulation of fluids and filled with Douglas fir wood cubes (2 × 2 × 2 cm) enclosed by a nylon net of 2-mm mesh, which excludes large sized predators and allows the colonization by metazoan larvae and juveniles (Gaudron *et al.*, 2010; Cunha *et al.*, 2013). The LD specimen was observed alive under a compound microscope. Specimens were preserved in 4% formaldehyde (2–4 h, gradient transfer to 96% ethanol).

MOLECULAR CHARACTERIZATION

DNA was extracted from one specimen from each site using the QIAamp DNA MicroKit (Qiagen). Fragments of three mitochondrial loci (encoding COI, NADH4, and 16S rRNA) and three nuclear loci encoding 18S, 28S rRNA, and histone 3 were amplified for phylogenetic analysis. Approximately 610 bp of COI, 650 bp of NADH4, 480 bp of 16S, 1650 bp of 18S,

1000 bp of 28S, and 300 bp of H3 was amplified using primers and the PCR amplification profiles are summarized in Table 1. PCR products were purified using a QIAquick Gel Extraction Kit (Qiagen) and sent for sequencing in both directions at GATC Biotech (UK). DNA sequences obtained during the present study were complemented with data from GenBank and available datasets (Lorion *et al.*, 2013; Thubaut *et al.*, 2013) (see Supporting information, Table S1) and aligned using CLUSTALW (Thompson *et al.*, 1997). Phylogenetic reconstructions were performed for single and concatenated genes using maximum likelihood and a general time reversible model with Gamma distribution of rates and a fraction of invariant sites using MEGA, version 6 (Tamura *et al.*, 2011).

The presence of bacteria was tested by PCR of fragments of genes encoding 16S rRNA, 23S rRNA, and APS reductase using methods described previously (Table 1) (Rodrigues *et al.*, 2013).

RESULTS

SHELL MORPHOLOGY

The largest specimen (length 2.13 mm) matched *I. argenteus* descriptions (Jeffreys, 1876; Dean, 1993; Oliver & Holmes, 2009; Ockelmann & Dinesen, 2011) of a delicate, iridescent, silvery-white, nacreous shell with a rounded oblong, submodioliform, plain edged outline, with the anterior slightly narrower than the posterior (Fig. 1). External sculpture of close-set commarginal lines (see Supporting information, Fig. S1), with short periostracal hairs over the posterior ventral area. Ligament thin, external, posterior to the beaks. Hinge plate minutely denticulate, posterior series of teeth twice the number of the anterior. Prodissoconch, two very large (549 µm), red–brown in colour. Juvenile specimens were similar to other small-sized bathymodiolins, although they were more oblong in form (Laming *et al.*, 2014).

PHYLOGENETIC RELATIONSHIPS

Sequences from LD and MAR specimens displayed between 99.4–99.9% similarity for each of the six studied genes. Phylogenies based on COI (Fig. 2A; see also Supporting information, Table S1 and Fig. 2S), on concatenated datasets of five genes (including closest relatives, Fig. 2B), and on all six genes (excluding *Idas washingtonius* and *Idas macdonaldi*; not shown) presented similar topologies and well-supported nodes using maximum likelihood methods. *Idas argenteus* was most closely related to *I. washingtonius* (K2P COI distance: 19.7% to 20.0%) and belonged to a clade that included not only *Idas* ESU O, *Idas* sp. P, *Idas* SAL1

Table 1. Primers and polymerase chain reaction parameters used for amplifications and sequencing reactions

	Gene	Annealing (cycles)	Primer names	Primer sequences (5'- to 3')	Reference
Host	COI mtDNA	50 °C (35)	H691	GTRTTAAARTGRCGATCAAAAAAT	Duperron <i>et al.</i> (2008)
			LCO1490	GGTCAACAAATCATAAAGATATTGG01	Folmer <i>et al.</i> (1994)
	NADH4 mtDNA	50 °C (35)	NADP2H	TGGAGCTTCTACGTGRGCTTT	Arevalo, Davis & Sites (1994)
			ArgBl	CAAGACCCCTTGATTTCGGCTCA	Bielawski & Gold (1996)
	16S rRNA	55 °C (35)	16SA	GGARGTASGCCCTGCCWATGC	Baco-Taylor (2002)
			LRJ	CTCCGGTTTGAATCAGATCA	Ratnasingham & Hebert (2007)
	18S rRNA	55 °C (35)	1F	ACCTGGTTGATCCTGCCAGTAG	Giribet <i>et al.</i> (1996)
			5R	CTTGGCAAATGCTTTTCGC	Giribet <i>et al.</i> (1996)
			3F	GTTTCGATTCCGGAGAGGG	Giribet <i>et al.</i> (1996)
			9R	ATCCTTCGCAGGTTACCTAC	Giribet <i>et al.</i> (1996)
Bacteria			Bi	GAGTCTCGTTTCGTTATCGGA	Okuzu <i>et al.</i> (2003)
			A2	ATGGTTGCAAAGCTGAAAC	Giribet <i>et al.</i> (1996)
	28S rRNA	55 °C (35)	C1prime	ACCCGCTGAATTTAAGCAT	Hassouna, Michot & Bachelierie (1984)
			C4	TCGGAGGGAACCAAGCTACTA	Hassouna <i>et al.</i> (1984)
	Histone 3	55 °C (35)	F1	ATGGCTCGTACCAAGCAGACVGC	Colgan <i>et al.</i> (1998)
			R1	ATATCCTTRGGCATRATRGTGAC	Colgan <i>et al.</i> (1998)
	16S	45 °C (27)	27F	AGAGTTTGATCATGGCTCAG	Lane (1991)
			1492R	GTTACCTTGTACGACTT	Lane (1991)
	23S	53 °C (35)	3505F	GACCGTCAGCTAAGGTCCCAA	Stewart & Cavanaugh (2009)
			4761R	CCAGTCAAACTACCCACCATG	Stewart & Cavanaugh (2009)
	APS reductase	58 °C (25)	APS1-FW	TGGCAGATCATGATYMYGG	Meyer & Kuever (2007)
			APS4-RV	GCGCCAAACYGGRCRTA	Meyer & Kuever (2007)
	V5-V6	45 °C (27)	V5-V6F	CAACACAGGATTAGATACCCCTG	Wang & Qian (2009)
			V5-V6R	TGTTGGGTTAAGTCCCGRAACG	Wang & Qian (2009)

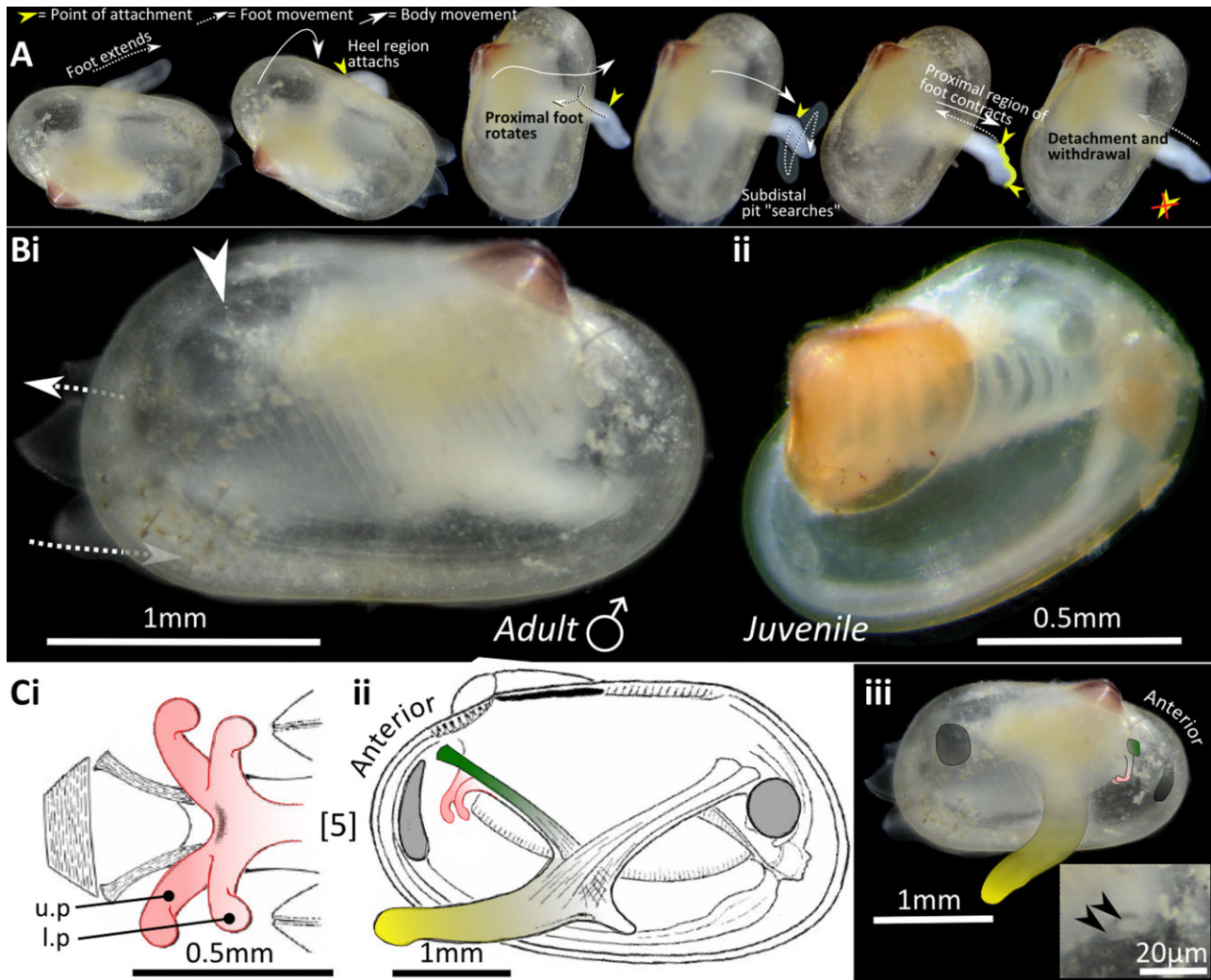


Figure 1. A) Locomotion in *I. argenteus* is driven by the extension, attachment and contraction of foot. B) Shell micrographs: i) adult male from Lacaze-Duthiers canyon; ii) juvenile from MAR. Dashed arrows: seawater current. Arrowheads: i) location of visibly-beating pericardium; C) comparative analysis of anatomy described in Ockelman & Dinesen (2011) adapted for Ci – ii, and Ciii) discernible anatomy through the shell of the adult male *I. argenteus*. Pink = labial palps, magnified and indicated by arrowheads in the inset of Ciii. Yellow = foot; black/grey = adductor muscles; green = anterior retractor bundle, or point of attachment of retractor, as in Ciii. (Colour version of figure available online.)

(Fig. 2), but also *Idas* ESU K and *Idas* ESU N (Fig. 2A; see also Supporting information, Fig. S2).

PCRs on all four bacterial genes using various DNA concentrations and PCR conditions, including nested PCR, failed to yield any product despite success with similar-sized symbiotic *Idas* spp. from the same experiment.

DISCUSSION

Idas argenteus were found to be associated with sunken wood in the colonization experiments performed at the Rainbow MAR vent field and in the LD

canyon. Although *Bathymodiolus azoricus* occurs at Rainbow and *Idas simpsoni* and *Idas modiolaeformis* are reported in LD (Génio *et al.*, in press), this is the first report of *I. argenteus* at these two sites.

The present study provides the first phylogenetic analysis of *I. argenteus*. Its closest relative is *I. washingtonius*, which is found in organic falls and cold seeps in the Eastern Pacific. Most of the other *Idas* species in the clade were found in the Western Pacific, mainly associated with wood. The only two Atlantic species (*I. macdonaldi* and *I. modiolaeformis*) displayed COI K2P distances with *I. argenteus* of between 19.3% and 20.4%, which in the upper range of distances measured among members of the clade

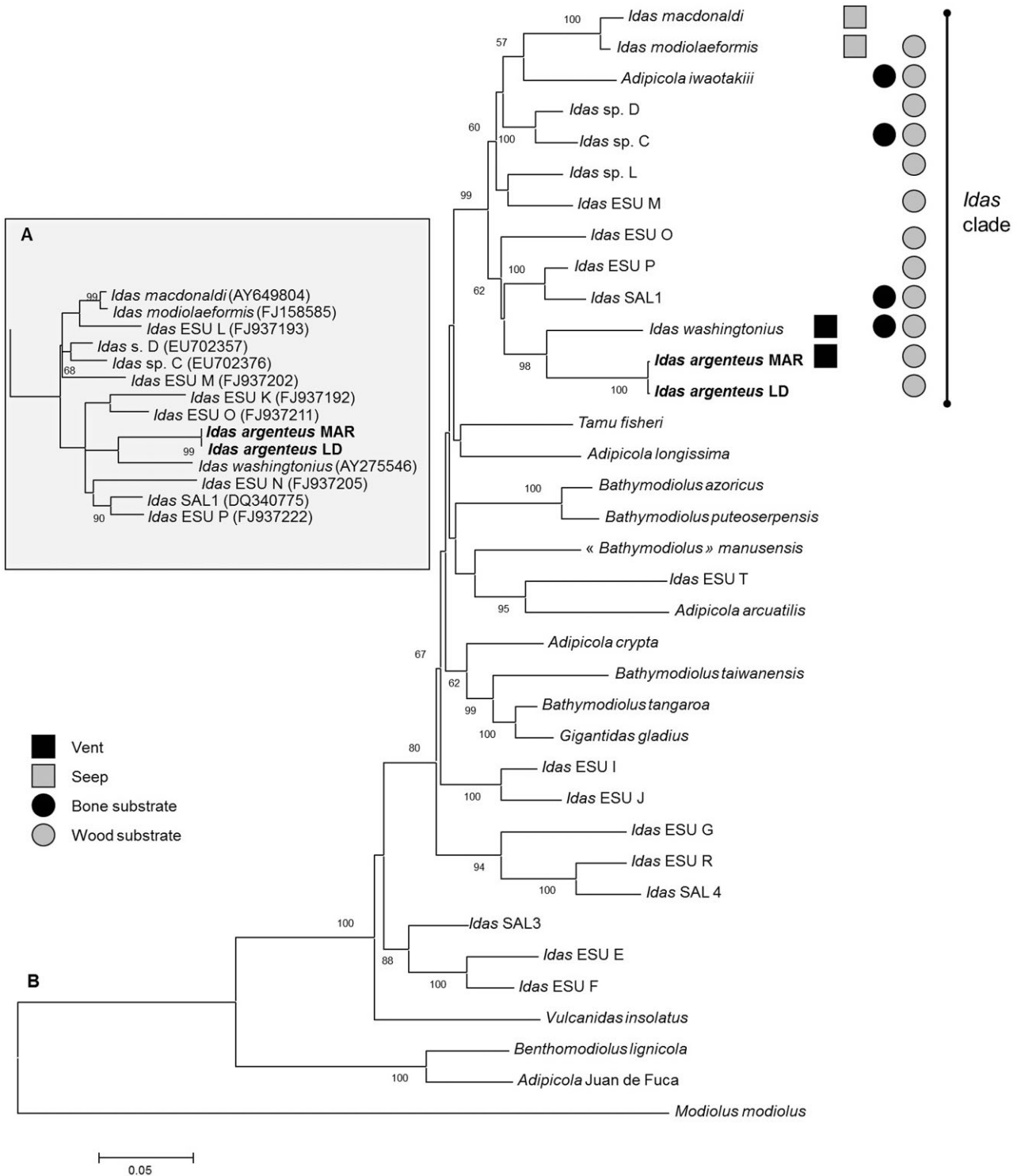


Figure 2. A) *Idas* clade detail of Maximum-likelihood (ML) tree obtained from the analysis of the Cytochrome Oxidase I (after Fig. S2). B) Phylogenetic relationships among Bathymodiolinae obtained by ML analysis of the multigene data set (2 mitochondrial and 3 nuclear genes) after Table S1. In both trees only bootstraps higher than 50% are given.

(9.3–23.1%). The results confirm that the genus *Idas*, as currently defined, is polyphyletic. Because *I. argenteus* is the type species of the genus, the use of the name *Idas* should be restricted to the species branching within the clade (*Idas* clade in Fig. 2) and other current *Idas* species should be renamed, as suggested in recent studies (Thubaut *et al.*, 2013).

Ockelmann & Dinesen (2011) reported no evidence of bacteria in the gills of *I. argenteus*. However, this could be the result of a site-specific rarity or the absence of symbionts. Without exception, symbiont acquisition in other bathymodiolin mussels occurs long before sexual maturation (Wentrup *et al.*, 2013; Laming *et al.*, 2014). The fact that symbiont genes were not detected by PCR tests in the specimens investigated in the present study is thus not size- or age-related because the type of fixation did not prevent getting PCR signals from bacterial symbionts in other studies (Rodrigues *et al.*, 2013). Even if present in small numbers, and below the detection limit of PCR approaches used in the present study, symbiont rarity would imply a negligible contribution to host nutrition. Further confirmation of the lack of symbionts using transmission electron microscopy or fluorescent *in situ* hybridization should be carried out when more material is available.

From the 13 *Idas* species belonging to the *Idas* clade in the COI tree (Fig. 2A; see also Supporting information, Table S1), symbiosis is documented in only five. *Idas macdonaldi*, *I. washingtonius*, *Idas* sp. C, and *Idas* sp. D have sulphur-oxidizing symbionts, whereas *I. modiolaeformis* has up to six different phylotypes with a relative dominance of methanotroph or thiotrophs depending on time of sampling or habitat (Duperron *et al.*, 2008; Lorion *et al.*, 2012; Rodrigues *et al.*, 2013; Thubaut *et al.*, 2013). Symbiosis in other species is not yet documented. At this stage, it can be assumed that the *Idas* clade had a common ancestor with thiotrophic symbionts, most likely extracellular, as in all species except *I. washingtonius* (Deming *et al.*, 1997). Extracellular thiotrophic symbionts were reported as an early acquisition dating back to the stem of the group, almost 30 Mya (Lorion *et al.*, 2013). Because *I. washingtonius* still harbours thiotrophic symbionts, it can be assumed that the loss (or considerable reduction) of symbiosis is a derived state and occurred within the branch leading to *I. argenteus*. A clock analysis using known calibrations (Lorion *et al.*, 2013) estimated that *I. argenteus* diverged from *I. washingtonius* between 11.1 and 13 Mya. Loss or reduction of symbiosis is not unexpected given the predisposition of *Idas* mussels for diverse, adaptable symbiont assemblages (Lorion *et al.*, 2012; Rodrigues *et al.*, 2013). An absence of symbionts in an otherwise symbiotic group has been observed in the Thyasiridae where some species have symbionts, whereas others do

not. A recent study even demonstrated that a single (potentially two cryptic) species may either harbour or lack symbionts in two distinct populations (Batstone *et al.*, 2014), a flexibility that could potentially also exist in deep-sea mussels.

The results reported in the present study concur with previous studies suggesting the absence of symbionts in *I. argenteus* (Ockelmann & Dinesen, 2011). To date, this is the only reported case of symbiosis loss or strong reduction within the bathymodiolin mussels clade. This shift to strict heterotrophy could be linked with the organic-enriched habitats, where larviphagy or filter-feeding may alone sustain the animal nutrition. Whether this will remain a unique example or represents a convergent trend in different mussel clades will require further investigation of symbiosis in smaller mussels.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Morphometrics and live observations of the adult male from Lacaze-Duthiers Canyon. A, interior view of the right valve. B, boxed region in A displaying larval shell and hinge plate: numbered arrowheads are the prodissoconch I, Iia, and Iib, respectively. The row of arrowheads denotes hinge-plate denticulation. C, exterior view of the left valve. D, E, dorsolateral and dorsal view of larval shell respectively. Numbered arrowheads are as shown in (B). F, G, left anterior region of the specimen observed during live dissection at two focal planes, details of the ventral 'feeding' groove and enlarged (for the genus) ciliated upper labial palp. Scale bars = 500 µm.

Figure S2. Maximum likelihood tree obtained from the analysis of the cytochrome oxydase I (COI) mitochondrial DNA data set (Table S1). Only bootstraps higher than 50% are given.

Table S1. Specimen collection sites and GenBank accession numbers for Mytilidae included in the phylogenetic analyses, *sensu* Lorion *et al.* (2013); Thubaut *et al.* (2013). Distribution: A, Atlantic; WP, Western Pacific; EA, Eastern Atlantic; EP, Eastern Pacific. Habitat: I, intertidal; W, wood; B, Bone; S, seep; V: vent. Genus1, as originally described; Genus2 as proposed (Thubaut *et al.*, 2013).

SHARED DATA

Sequences have been deposited in GenBank (accession numbers: LM992891–LM992902).